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DOCUMENT-IDENTIFIER: US 6589730 B1
TITLE: Methods for identifying protein-protein interactions by selective transduction

Brief Summary Text (4):
Bacteriophage expressing a peptide on its surface has been used to identify protein binding domains, including antigenic determinants, antibodies that are specifically reactive, mutants with high affinity binding, identify novel ligands, and substrate sites for enzymes. In its most common form, a peptide is expressed as a fusion protein with a capsid protein of a filamentous phage. This results in the display of the foreign protein on the surface of the phage particle. Libraries of phages are generated that express a multitude of foreign proteins. These libraries are bound to a substrate or cell that presents the binding partner of interest. This screening process is essentially an affinity purification. Bound phage are recovered, propagated, and the gene encoding the foreign protein may be isolated and characterized. This technology is commonly referred to as "phage display."

Drawing Description Text (3):
FIGS. 1A and 1B are schematic representations of phage vectors for mammalian cell transduction. FIG. 1A depicts the parent phage vector with wild type pIII coat protein. The base vector is M13 genome with ampicillin resistance (Amp.sup.R) gene and GFP expression cassette inserted into the intergenic region between pIV and pII (MEGFP3). The MEGFP3 vector contains the following elements: ori-CMV, SV40 replication origin and CMV promoter; EGFP, enhanced green fluorescent protein gene; BGH, and a bovine growth hormone polyadenylation sequence. FIG. 1B represents the FGF-pIII fusion display phage (MF2/1G3).

Drawing Description Text (7):
FIG. 5 is a bar graph representing the transduction of COS cells by peptide display phage.

Detailed Description Text (2):
As noted above, the present invention provides a method of ligand display that identifies and/or selects for cells or tissue types that bind ligands as well as allowing identification of protein ligands that bind and internalize on the basis of expression of a transgene that is carried on a ligand displaying genetic package construct. While it should be understood that a variety of ligand display methods may be utilized (e.g., phage display, RNA-peptide fusions, and ligand displaying bacteria), the present invention uses bacteriophage ligand display to exemplify the various embodiments.

Detailed Description Text (5):
A variety of displaying genetic packages may be used within the context of the present invention. A "ligand displaying genetic package" as used herein, refers to any package which comprises a peptide/protein ligand and carries an expressible nucleic acid molecule for detection, once internalized in the target cell. For example, display may be by a virus, RNA-peptide fusions, bacteriophage, bacteria, or similar system (See, Kay, Phage Display of Peptides and Proteins, pages 151-193, Academic Press, 1996). Preferred methods utilize bacteriophages. Such phage include the filamentous phages, lambda, T4, MS2, and the like. A preferred phage is a filamentous phage, such as M13 or f1.

Detailed Description Text (7):
In the preferred filamentous phage system, a wide range of vectors are available (see, Kay et al., Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, San Diego, 1996). The most common vectors accept inserts in gene III or gene

VIII. Furthermore, the foreign gene can be inserted directly into the phage genome or into a phagemid vector. Methods of propagation of filarnentous phage and phagemids are well known.

Detailed Description Text (13):
Phage display libraries of random or selective mutations of known ligands for improved gene delivery are performed in the same manner as described for screening random peptide libraries such libraries are referred to herein as a "mutein library" (i.e., a library of selective or random mutations). Random mutations of the native ligand gene may be generated using DNA shuffling as described by Stemmer (Stemmer, Nature 370: 389-391, 1994). Briefly, in this method, the ligand is amplified and randomly digested with DNase I. The 50-300 base pair fragments are reassembled in an amplification performed without primers and using Taq DNA polymerase or similar enzyme. The high error rate of this polymerase introduces random mutations in the fragments that are reassembled at random thus introducing combinatorial variations of different mutations distributed over the length of the gene. Error prone amplification may alternatively be used to introduce random mutations (Bartell and Szostak, Science, 261:1411, 1993). The ligand may be mutated by cassette mutagenesis (Hutchison et al., in Methods in Enzymology 202:356-390, 1991), in which random mutations are introduced using synthetic oligonucleotides and cloned into the ligand to create a library of ligands with altered binding specificities. Additional mutation methods can be used. Some additional methods are described in Kay et al., supra. Further, selective mutations at predetermined sites may be performed using standard molecular biological techniques (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989).

Detailed Description Text (22):
Thus, an example of a dual display filamentous phage presents a ligand (e.g., FGF) as a fusion to gene III and an endosomal escape peptide fused to gene VIII. The locations of the ligand and escape sequences are interchangeable. Escape sequences that are suitable include, without limitation, the following exemplary sequences: a peptide of Pseudomonas exotoxin (Donnelly, J. J., et al., PNAS 90:3530-3534, 1993); influenza peptides such as the HA peptide and peptides derived therefrom, such as peptide FPI3; Sendai Virus fusogenic peptide; the fusogenic sequence from HIV gp1 protein; Paradaxin fusogenic peptide; and Melittin fusogenic peptide (see WO 96/41606).

Detailed Description Text (25):
As described herein, the library is then propagated in the display phage by transfection of a suitable bacteria host (e.g., DH5.alpha.F' for filamentous phages), and growing the culture, with the addition of a replication-competent helper virus if necessary, overnight at 37.degree. C. The phage particles are isolated from the culture medium using standard protocols.

Detailed Description Text (26):
Infection of mammalian cells with phage is performed under conditions that block entry of wild type phage into cells (Barry et al., Nature Med. 2:299-305, 1996). Phage are added directly to cells, typically at titers of .ltoreq.10.sup.12 CFU/ml in a buffer, such as PBS with 0.1% BSA or other suitable blocking agents, and allowed to incubate with the cells at 37.degree. C. or on ice. The amount of phage added to cells will depend in part upon the complexity of the library. For example, a phage display library containing 10.sup.5 members has each member represented 10.sup.6 times in 1 ml of a typical phage titer of 10.sup.11 colony forming units/ml.

Detailed Description Text (28):
The phage display library is ultimately screened against the target tissue or cell line. Screening can be performed in vitro or in vivo. While combinatorial screening methods have been performed in the past, these methods are unable to determine the transduction capability of the displayed ligand (see, U.S. Pat. No. 5,733,731, incorporated herein by reference). The criteria for a positive "hit" in the present invention is that the phage must be able to bind, be internalized, and express the genomic DNA containing the reporter gene in the target cell. In this regard, it is believed that the phage should bind, internalize, translocate to the nucleus, uncoat and replicate, in order to express the gene. Thus, only phage that express a reporter gene are selected.

Detailed Description Text (31):
Screening may be performed directly against the target cells with no pre-screening or pre-enrichment. In one aspect, the present invention provides a method of identifying target cells or tissues for known or putative ligands. In this regard, phage display may be used to display a library of known or putative ligands) (e.g., peptides, antibody

fragments and the like) and screen singular tissues or cell types, or pools of tissues or cell types, thereby identifying target cells or tissues which are effectively transduced by a ligand. As used herein, "pool" refers to two or more cell types or tissue types. In one embodiment, known ligands are presented on a ligand displaying genetic package to a pool of a variety of cell or tissue types and transgene expression is monitored. In a further embodiment, putative ligands are used to screen a pool of a variety of cell or tissue types for transduction ability. In this regard ligands may be recovered and identified which efficiently transduce a particular tissue or cell type. Identification of cell specific ligands could greatly improve existing vectors for therapeutic gene delivery by targeting specific cells thus reducing toxicity and allowing vectors to be administered systemically.

Detailed Description Text (33):
In one aspect, the present invention may be utilized to identify a variety of protein-protein interactions. In particular, a set of unknown proteins/peptides may be selected based upon interaction with another set of known or unknown proteins/peptides (e.g., random peptides, cDNA libraries, or antibody gene libraries). In one embodiment, putative ligands are displayed on the surface of filamentous phage that carry a reporter gene. These display phage are contacted with a cell line displaying a putative anti-ligand (protein/peptide) on its surface as a receptor fusion protein, such that binding of successful detection of the reporter gene requires binding of the phage display ligand and the cell surface displayed anti-ligand, as well as internalization and transgene expression. Such screening can be utilized in a variety of methods, for example, a known ligand may be screened against a library of potential anti-ligands, a library of unknown ligands may be screened against a known protein/peptide anti-ligand, and two libraries of peptides/proteins may be screened against each other to identify ligand/anti-ligand interactions (protein-protein).

Detailed Description Text (36):
In a further embodiment, a large pool of cDNAs may be tested by transfecting into a large number of mammalian cells (e.g., COS cells). Ligand displaying phage are exposed to the transfected cells and positive cells identified by either drug selection or detection of an expressed transgene (e.g., GFP sorted by FACs). PCR may be performed on single cells to identify ligand/anti-ligand binding pairs. In this regard PCR primers directed to the known portion of the fusion construct may be used. For example, for phage display using pill to display the ligand, the PCR primer will be directed to the pill gene, while in order to identify the anti-ligand, the PCR primer will be directed to the surface membrane protein (e.g., a receptor domain) encoding portion of the fusion construct. Alternatively, the plasmids within positive cells may be rescued by Hirt supernatant method and separated from phage DNA by gel electrophoresis or chromatography. (Kay et al., supra). The selected cDNA plasmids may then be used to retransform bacteria. New plasmid DNA is prepared and used for additional rounds of screening by transfection into the cells and phage contact.

Detailed Description Text (37):
In a further embodiment, known or putative ligand-display phage may be used to screen a panel of cells that each express a potential target receptor. The source of the target receptor may be a known (i.e. cloned) receptor cDNA, or a collection of putative receptor cDNAs. For example, the putative receptor cDNAs may be identified from an epitope-tagged cDNA library as cDNAs that encode proteins that appear on the surface of cells. (see, Sloan et al., Protein Expression and Purification 11:119-124, 1997). Such cDNAs are inserted into an appropriate mammalian expression vector and transfected into a host cell. Preferably the host cell is eukaryotic, and more preferably the host cell is mammalian. The expression of the cDNA may be either stable or transient. Following expression the cells are contacted with the ligand-display phage and monitored for transgene expression (e.g., drug resistance, GFP, or other detectable product). One skilled in the art would recognize that identification of cell or tissue types as described above, in addition to using ligand display phage, could also performed by utilizing other ligand displaying means, such as RNA-peptide fusions as described by Roberts and Szostak (Proc. Nat. Acad. Sci. USA 94:12297-12302, 1997), other phage types, or on bacteria.

Detailed Description Text (41):
Alternatively, multiple rounds of infection and selection are performed to reduce the complexity of the infecting phages. For example, drug-resistant colonies are pooled and the selected inserts amplified and cloned back into the phage display vector for a new round of infection. When the reporter is fluorescent, flow cytometry can be used to select the strongest fluorescing cells to select the most highly efficient gene delivery ligands. More stringent screening conditions also include higher selective

drug concentrations. At the completion of a selection process, representative phage clones may be subjected to DNA sequence analysis to further characterize gene delivery ligands.

Detailed Description Text (46):
Screening in vivo may be performed similar to methods for targeting organs or xenograft tumors using phage displayed peptides (Pasqualini et al., Nature Biotech. 15: 542-546, 1997; Pasqualini et al., Nature 380: 364-366, 1996), except that the tissues, organs, or tumors are examined for reporter gene expression instead of the presence of phage. Briefly, a phage display library is injected intravenously into animals, generally mice, and organs or tumor samples are tested for reporter gene function at 48-96 hours after injection. Tumor cells may be cultured in selective conditions or sorted by flow cytometry or other method to enrich for cells that express the phage transducing gene. The ligand encoding sequences can be amplified from selected cells as described above. As in in vitro screening, repeated rounds of infection and rescreening, alone or in combination with increased screening stringency, may be used to obtain the most efficient gene delivery ligands.

Detailed Description Text (69):
A mammalian expression cassette is inserted into a phage or phagemid vector and is used to detect ligand mediated phage entry via reporter gene expression in mammalian cells. A type 3 filamentous phage vector is modified for transduction of mammalian cells by insertion of a GFP expression cassette consisting of a CMV mammalian transcriptional promoter, the green fluorescent protein gene from pEGF-PN1 (Clontech; Palo Alto, Calif.), and a bovine growth hormone transcriptional terminator and polyadenylation signal to make the vector, MEGFP3 (see FIG. 1A). The mammalian expression cassette also contains an SV40 origin of replication adjacent to the CMV promoter. Similar constructs for monitoring entry and subsequent expression of phage genomes in mammalian cells are constructed from other known phage or phagemid vectors including pCANTAB 5 E (Pharmacia Biotech; Piscataway, N.J.) or M13 type 3 or 33 for gene III fusions (see Kay et al., Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, 1996; McConnell et al., Mol. Divers. 1:165-176,1996) and M13 type 8 or 88 vector for fusions to gene VIII protein (Roberts et al., Methods Enzymol. 267:68-82, 1996; Markland et al., Gene 109:13-19, 1991).

Detailed Description Text (71):
Construction of FGF2-Containing Phage Display Vectors

Detailed Description Text (72):
In the following examples, a phage that displays FGF2 on its surface is used to bind to the FGF2 receptor on mammalian cells and be internalized. An FGF2 gene is subcloned into the modified M13 phage type 3 vector, MEGFP3, to create the ligand display phage, MF2/1G3 (see FIG. 1B). The gene may also be mutated such that it encodes an FGF2 (C96S) (C78S) double mutant which enhances expression efficiency. The MEGFP3 vector has been modified with a mammalian expression cassette designed to express the reporter gene GFP to monitor mammalian cell transduction by the phage. Other vectors include pCANTAB 5 E (Pharnacia Biotech; Piscataway, N.J.) or M13 type 3 or 33 for gene III fusions (see Kay et al., Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, 1996; McConnell et al., Mol. Divers. 1:165-176,1996). Similarly, FGF2 is cloned into M13 type 8 or 88 vector for fusion to gene VIII protein (Roberts et al., Methods Enzymol. 267:68-82, 1996; Markland et al., Gene 109:13-19, 1991).

Detailed Description Text (78):
To increase the sensitivity of the assay for transduction by ligand display phage the target cell line is transfected with a plasmid that is designed to express the SV40 large T-antigen (i.e. pSV3neo). This plasmid also contains a drug selection gene such as neomycin phosphotransferase (neo) which confers resistance to the antibiotic G418 in stabley transfected mammalian cells. Following transfection of the target cell line with plasmid DNA using standard methods (i.e. CaPO.sub.4 co-precipitation) the cells are split and maintained in G418 containing media until drug resistant colonies appear. The colonies are expanded to test for SV40 T-antigen synthesis by western blotting or immunoprecipitation using a suitable antibody. Examples of T-antigen expressing target cell lines are: BOS (BHK with SV40 T-Ag) for screening FGF variants; HOS-116 (HCT116 with SV40 T-Ag) for screening peptides that target human colon carcinoma; AOS-431 (A431 with SV40 T-Ag) for screening EGF variants (all parent cell lines are available from ATCC, Manassas, Va.)

Detailed Description Text (84):
Transductiol of Mammalian Cells by FGF2-Ligand Display Phage

Detailed Description Text (85):
FGF2 display phage (MF2/1G3) and an identical phage that lacks the FGF2 gene (MEGFP3) are compared for receptor mediated internalization and reporter gene expression in COS cells. The phage are incubated with the cells for 4 hours at 37.degree. C. in DME (Dulbecco's modified Eagles medium, Life Technologies (Gibco BRL); Rockville, Md.) containing 2% BSA (bovine serum albumin) as a blocking agent. After washing to remove unbound phage the cells are returned to the incubator for an additional 72 hours. Transduction is measured by counting GFP positive autofluorescent cells. As shown in FIG. 4B, the FGF2 display phage result in about a 10 fold greater transduction efficiency than the control phage indicating that the displayed FGF2 ligand on the surface of the phage particles results in receptor mediated binding and internalization of phage with subsequent expression of the phage reporter gene. The specificity of the FGF2-phage mediated transduction is demonstrated by successful inhibition of transduction with excess free FGF2 (2 .mu.g/ml) (FIG. 4B). The low level nonspecific uptake and transduction by the control phage (MEGFP3) is not affected by the presence of excess FGF2.

Detailed Description Text (86):
It is important to show that the MEGFP3 control phage is equally capable of transducing mammalian cells as the display phage when appropriately targeted. To compare the transduction ability of both the FGF2-phage and the control phage, equivalent titers of each phage were used to transfect COS cells using a avidin-biotin FGF2 targeting method. In this method biotinylated FGF2 is contacted with the cells and used to capture phage particles via the addition of avidin and a biotinylated anti-phage antibody. The phage/FGF2/cell binding is performed on ice, unbound phage removed by washing, cells returned to the incubator at 37.degree. C., and transduction assessed at 72 hours. As seen in FIG. 4A, there is no significant difference in transduction between FGF2-phage and control phage when FGF2 is attached to the phage via an avidin biotin linkage. In this case the biotinylated FGF2 is in excess of the FGF2 displayed on the phage surface such that internalization is expected to be primarily via the biotinylated FGF2. These data demonstrate specific receptor mediated transduction of mammalian cells by filamentous phage that genetically display a targeting ligand (FGF2).

Detailed Description Text (88):
Constructio of a Reporter Gene and a Drug Resistance Gene in Phage Display Vectors

Detailed Description Text (92):
A M13 phage display library of random or unknown sequences is spiked with pFGF-maM13 phage. The mixture is used to infect COS cells as described above. The cells are washed extensively to remove non-specifically bound phage. Cells are replated 48-96 hours later at a 1 to 10 dilution and grown in G418 to select only cells that receive the transducing phage gene. Alternatively, the GFP expressing cells are isolated by flow cytometry using an excitation wavelength of 488 and emission wavelength of 510.

Detailed Description Text (112):
Peptides are selected which have been previously identified from a random library by one or more panning or screening procedures using conventional vectors and panning methods (see Kay et al., Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, 1996). The DNA encoding the selected peptides is inserted as a fusion to the pIII coat protein in the MEGFP2 vector containing the GFP reporter gene cassette.

Detailed Description Text (113):
An M13 phage random peptide library is screened for peptides that bind and internalize in an FGF receptor overproducing cell line, Flg37 (an FGFR1 stable transfectant of L6 cells (available from the ATCC; Manassas, Va.) obtained from Dr. Murray Korc, UCI; Irvine, Calif.). In addition, such a cell line may be easily created by those skilled in the art. Following 5 rounds of panning and rescreening the complexity of the library is reduced such that 80% of the phage are represented by a single peptide-pIII fusion. The resulting peptide, FL5, has the sequence FVPDPYRKSR (SEQ ID NO: 1). The same library is also screened against Flg37 cells by selecting infective phage particles that internalize and associate with nuclei and cytoskeletal proteins. The 2 predominant peptide sequences identified by this screen after 5 rounds of panning are FN5A, CGGGPVAQRC (43%) (SEQ ID NO: 2) and FN5B, CLAHPHGQRC (34%) (SEQ ID NO: 3).

Detailed Description Text (123):
To accomplish this, a library of cDNAs is inserted into a mammalian expression vector

(pcDNA 3.1) such that the cDNAs are fused to the transmembranes and intracellular domains of EFG receptor cDNA. DNA is prepared from individual or pools of bacterial clones that have been transformed to carry the cDNA-receptor fusion protein expression plasmid. COS cells are transfected with the resulting plasmid DNAs in six well plates at low density. At 24 hours later, ligand display phage carrying the CMV driven reporter gene GFP are added to the transfected COS cells.

Detailed Description Text (124):
Binding of the phage displayed ligand to the cell surface display binding target (i.e. protein--EGF receptor fusion protein), results in dimerization of the receptor and subsequent internalization of phage that display the binding ligand. The internalized phage are trafficked to the nucleus where the reporter gene is expressed. 72 hours after adding phage, cells expressing the reporter gene are selected by FACs. cDNAs encoding reactive peptides are identified by the presence of GFP positive cells in the COS transfectants for each cDNA or cDNA pool. The binding ligand is identified by PCR amplification and sequencing of the phage ligand-pIII fusion gene. The target peptide is identified by PCR amplification and sequencing the peptide-EGF receptor fusion protein from the selected cell(s).

Detailed Description Text (127):
Phage that display a ligand as a pIII fusion on the phage coat and carry the GFP expression cassette are prepared using standard protocols, as discussed above. Control phage that carry GFP but don't display a ligand are also prepared. Candidate cell targets are seeded into 6 well culture plates at about 40,000 cells/well. At 24 hours after seeding cells, phage are added at .about.10.sup.10 pfu/ml. The plates are incubated at 37.degree. C. for an additional 72 hours. Each cell well is scored by counting GFP positive autofluorescent cells. The cell types that have a ratio of GFP positive cells in the ligand-phage treated well/control phage treated cells of greater than 1.0 are selected as targets for further study and characterization. As an alternative to GFP, a drug resistance gene can be used in which case after 72 hours the cells are allowed to continue growth in selective medium containing the drug. Positive cell types are scored by counting wells that have drug resistant colonies.

Detailed Description Text (131):
Ligand display phage are constructed as discussed above, with the ligand being full-length or fragments of coat or envelope proteins of a known or suspected pathogen. The ligand expressed on the display phage coat can be expressed from the cDNA or cDNA derivative of the coat or envelope protein of a known or suspected pathogen (e.g., HIV envelope protein gene). The envelope gene is randomly fragmented to form a library of display phage display distinct portions of the coat protein. Thereby allowing determination of the portion of the gene that encodes a protein that functionally interacts with the host cell surface receptors allowing internalization. Smaller pathogen coat proteins are displayed in entirety. The pathogen coat display phage acts as surrogate pathogen with the advantage of providing a simple assay for detection of host cells. Phage displaying coat protein are screened against various cell types in vitro as described above or in vivo by injection and subsequent identification of target cells and tissues by fluorescent microscopy, FACS analysis to detect GFP, or growth in selective medium to detect expression of a drug resistance marker.

Detailed Description Text (134):
The gene(s) or portion of a gene that interacts with the host cell surface receptor to allow internalization is identified by making a phage display library of the cDNAs expressed by the pathogen or of the pathogen genome or fragments of the genome. The display library phage vector carries the GFP or suitable reporter gene driven by the mammalian promoter, as described above. The libraries are then screened against a known or putative host cell types by detecting transgene expression (i.e., drug selection or other detectable marker). Once cells are identified, the sequence of the nucleic acid encoding the internalizing ligand is determined by PCR sequencing of the pIII-putative ligand fusion construct.

Other Reference Publication (3):
Larocca et al., "Targeted Gene Delivery to Mammalian Cells Via Fibroblast Growth Factor (FGF-2) Display Phage," Cancer Gene Therapy 5(6): Abstract No. PD-31, p. S10, 1998.

Other Reference Publication (6):
Pasqualini and Ruosiahti, "Organ Targeting in vivo Using Phage Display Peptide Libraries," Nature 380:364-366, 1996.

Other Reference Publication (17):

Sawyer et al., "Methodology for selection of human antibodies to membrane proteins from a phage-display library," Journal of Immunological Methods 204: 193-203, 1997.